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Serological and Histological Findings in Infection and Transmission of GBV-C/HGV to Macaques

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Seven healthy macaques were inoculated with the GBV-C/HGV-RNA serum from a non-A-E hepatitis patient. The serology and pathology of the liver in the animals were observed. The results indicated that all inoculated animals were infected with a GBV-C/HGV-RNA viremia and had mildly abnormal alanine transaminase levels during the infectious period. The histology, immuno-histochemistry, and *in situ* hybridization in the liver tissues of the inoculated animals also showed that there was a very mild hepatitis with the positive antigenic expression and the genome of GBV-C/HGV-NS5 in hepatocytes. The pathological changes in the infected animals appeared to become normal whether or not GBV-C/HGV-RNA viremia persisted. There is a possibility that the mild virulence of the GBV-C/HGV to the host became harmless with time after inoculation. Infection and the transmission of the GBV-C/HGV virus in the macaques provides an appropriate animal model and new information about GBV-C/HGV infection in both humans and animals. It is possible that this virus is a mild and self-limited pathogenic agent to the hepatic cells of primates. ***J. Med. Virol. 60:28–33, 2000.***

KEY WORDS: GBV-C/HGV; macaques; infection and transmission models; mild and self-limited hepatitis

INTRODUCTION

Hepatitis C virus (HCV) is responsible for most cases of post-transfusion non-A, non-B hepatitis. It has become increasingly evident, however, that there are patients with non-A, non-B hepatitis who are not infected with HCV and other known hepatitis viruses. There remains a residual risk of post-transfusion hepatitis.

Simons et al. [1995a, 1995b] and Kim et al. [1995] reported an identical novel RNA virus: GB virus C (GBV-C) and hepatitis G virus (HGV), respectively, from patients with non-A-E hepatitis in different areas, and considered that this GBV-C/HGV might have been responsible for the non-A-E post-transfusion hepatitis [Kim et al., 1995; Simons et al., 1995; Linnen et al., 1996]. GBV-C/HGV RNA was also detected in the sera of Chinese patients who had the non-A-E hepatitis or who were co-infected mostly with HCV, hepatitis B virus (HBV), and so on [Boan et al., 1996; Ren-Feng et al., 1996; Yun et al., 1996; Yusen et al., 1996]. It is not known whether GBV-C/HGV is a new pathogenic agent for hepatic cells or harmless [Alter, 1996; Loya, 1996].

In this report, a group of macaques was used for infection and transmission with a serum from a non-A-E hepatitis patient with GBV-C/HGV-RNA. The serology and the hepatic pathology of the experimentally inoculated animals were observed. The results provide additional information that the GBV-C/HGV may be a mild and self-limited hepatitis virus.

MATERIALS AND METHODS

Serum Sample

Serum from a non-A-E hepatitis patient was used for inoculation. The GBV-C/HGV RNA was detected by reverse transcription-nested polymerase chain reaction (RT-PCR) for the partial gene of NS5 and its nucleotide sequence was analyzed [Boan et al., 1996; Yun et al., 1996]. Serum from healthy volunteer blood, which was negative for the known hepatitis viruses, was a negative control for inoculation of a control macaque.

Animals

Eight macaques about 3 years old used for the transmission of infection were secured through the Center of Experimental Research for Animals of the Chinese Academy of Medicine. All animals were maintained and monitored at the Center of Animal Research in our Institute based on the requirements for human care

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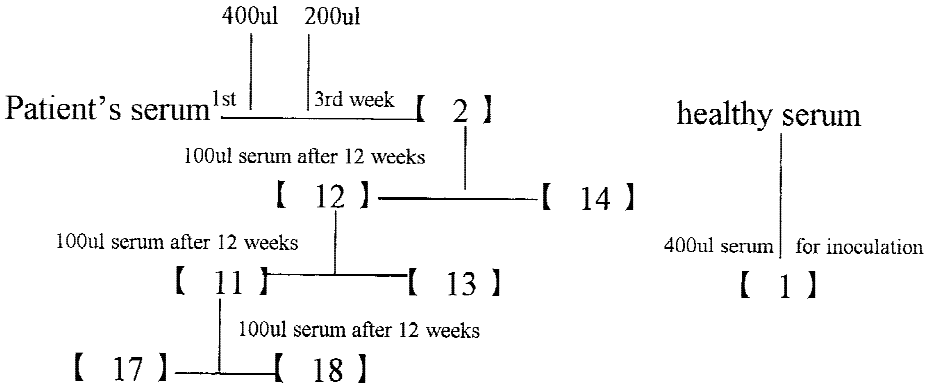

Fig. 1. Flow diagram of GB virus C/hepatitis G virus (GBV-C/HGV) macaques transmission experiments. The numbers in brackets indicate the name of the inoculated macaques. The amount of serum used for the inoculation and the inoculated time is indicated; the macaques were inoculated intravenously.

and the ethical use of primates in an approved facility. The diagram used for serial inoculation to the seven macaques and the control is shown in Figure 1. The animals had not been inoculated intravenously with any material from animals or humans before this study.

Sera and hepatic biopsies from each inoculated animal were obtained 2 weeks before inoculation and weekly/bi-weekly (sera) or 5, 36, 72 weeks (hepatic tissues) after inoculation under general anesthesia. The serum levels of alanine aminotransferase (ALT), serum GBV-C/HGV RNA, and anti-E2, and the changes in histology, immuno-histochemistry, and in situ hybridization in hepatic tissues from those inoculated animals were monitored.

Detection of Viral RNA

GBV-C/HGV RNA were detected by RT-nested PCR [Boan et al., 1996]. The total RNA was extracted from 50 μl serum by a modified method of Boom et al. [1990]. The ethanol-washed and dried nucleic acids were suspended in 30 μl mixture of the RT reaction: 1 mmol/L dNTPs, 20 U Rnasin, 5 U AMV reverse transcriptase (Promega), and 1 μmol/L of each outside sense and anti-sense primer. RT was carried out at 42°C for 30 min and inactivated by heating 5 min at 95°C.

The 5-μl mixture of RT was added into 20 μl PCR reaction mixture for the first round of PCR. The first round of PCR mixture contained 1.5 mmol/L $MgCl_2$, 1 U Taq polymerase (Promega). Thirty cycles of PCR were performed (95°C 1 min, 55°C 1 min, and 72°C 1.5 min). Then 2.5 μl of first round of PCR product were added into 22.5 μl of the second round of PCR mixture containing 1.5 mmol/L $MgCl_2$, 40 μmol/L dNTPs, 0.2 μmol/L of each inner sense and anti-sense primer; 1 U Taq polymerase PCR was performed for another 30 cycles (95°C 1 min, 61°C 1 min, 72°C 1 min). PCR products were then analyzed on 1.5% agarose gels using PGEM-7Zf/HaeIII marker as standard and stained with ethidium bromide.

For the first round of PCR, the outer primers were sense: 5′GAGGTGTTCTTCAAAGACCG3′ (7734–7753nt), anti-sense: 5′GCTACTGTCGAAGCA(A/G)GTGG3′ (7961–7980). For nested PCR, the primers were sense: 5′GGACTTCCGGATAGCTG3′ (7796–7812), and anti-sense: 5′GC(A/G)TCCACACAGATGGCGCA3′ (7941–7960). The target fragment amplified by using the nested primers was 165 bp.

Monoclonal Antibody 2B11-B12

The amino acid sequence of a synthetic peptide derived from the NS5 of GBV-C/HGV was PHAAMGWGSKVSVKDLATPAGKMAVHDRL (2381–2410 residues). The peptide 29-residues (P29) were prepared by solid-phase peptide synthesis on PE 431A and purified. The purified P29 was coupled to bovine serum albumin as the immunogen and then, the Balb/c mice were immunized according to the method described by Boudet et al. [1995].

The monoclonal antibody (MAb) 2B11-B12 was developed by using standard hybridization techniques [Galfre and Milstein, 1981]. 2B11-B12 belongs to a subclass IgG1 and its relatively affinity is about 0.2 μg/ml. Its specific binding activity to the P29, which was coated on solid-phase, was determined by enzyme-linked immunosorbent assay (ELISA). There was no binding activity to the antigens of A-E hepatitis virus ELISA [Jin et al., 1997; Shen et al., 1998]. 2B11-B12 was used for detecting the NS5 antigens expressed in the hepatocytes of the macaques in the immunohistochemistry.

Immuno-Histochemistry

The expressed NS5 antigens of GBV-C/HGV in hepatic tissues from macaques were detected by using the 2B11-B12 MAb and the commercial LSAB methods (Dako Co.). The protocols were based on the description of the LSAB kit. Paraffin-embedded sections were dewaxed normally and then digested 45 min at 30°C by 0.1% trypsin (GIBCO; Gaithersburg, MD). The anti-NS5 MAb 2B11-B12 (50 μg/ml), biotinylated-anti-mouse IgG, conjugate of streptavidin-labeled horseradish peroxidase (HRP), and the substrate H_2O_2-diamino benzidine (DAB)/ACE were added to the slides step by step based on the protocols of the LSAB kit.

In Situ Hybridization

The purified partial cDNA fragments of GBV-C/HGV-NS5 were used as the probes labeled with digoxigenin, and the genome of the NS5 of the GBV-C/HGV

in the hepatic cells was detected by in situ hybridization. The PCR products of GBV-C/HGV-NS5 were purified by using the Wizard PCR Preps kit (Promega) and sequenced for preparing the probes. The probes were 165 nt of length within 7796–7960 nt of NS5 region. The cDNA sequences of the probes were 5′-GGACTTCCGGATAGCTGAGAAGCTTATCCTGGGAGACCCGGGGCGGGTGGCCAAGGCGGTGTTGGGGGGGGCTTACGCCTTCCAGTACACCCCAAATCAGCGAGTTAAGGAGATGCTCAAACTGTGGGAGTCAAAGAAAACACCTTGCGCCATCTGTGTGGACGC-3′ [Yun et al., 1996]. In situ hybridization was completed according to the protocols in DIG DNA Labeling and Detection kit (Boehringer Mannheim). The 8-μm thick paraffin-embedded sections were dewaxed with xylene and ethanol, fixed with 4% paraformaldehyde/0.1 M phosphate-buffered saline (PBS) pH 7.2 (fixative solution). The slides were rinsed with 0.1 M PBS, pH 7.2, 0.1 M glycine/0.1 M PBS, 0.4% Triton x-100/1 M PBS in series. Then the slides were digested with the 1.5 μg/ml of proteinase K. The slides were rinsed with fixative solution, 0.1 M PBS, 0.25% acetic anhydride, and 2 × SSC in series. The slides were covered with 80–100 μl of hybridization solution containing 5% formamide, 0.1% laurouyl sarcosine, 0.02% SDS, 0.2% blocking reagent, 250 μg/ml DNA of salmon sperm, and 0.5 pmol/ml denatured DIG-labeled probe. The slides were incubated with gentle agitation for 16 hr. After stringent washing, the sections were incubated in blocking solution, in anti-DIG-AP conjugate solution, in detection buffer and in color solution (NBT/BCIP) in series. The slides were immersed in xylene, washed, and dried. The results were documented under a light microscope.

Detection of Anti-E2

The amino acid sequences derived from the E2 region of GBV-C/HGV were synthesized by the solid-phase peptide synthesis on PE 431A and purified. The sequence of P32 peptide was VRRCSELMGRRNRVCPGFAWLSSGRPDGFIHD (625–656 residues in E2). The ELISA for detecting the anti-E2 was established and optimized. The 100 μl macaque sera were diluted 1:10 and incubated in a polystyrene microplate well coated with P32 (4 μg/ml). After washing, the bound macaque IgG was detected by anti-human IgG-HRP conjugate. Tetramethylbenzidene (TMB) solution containing hydrogen peroxide was added to the wells. The OD values at 450 nm were read on the Σ 960 EIA photometer. Samples with absorbance values lower than the cut-off value were negative for anti-E2, samples with absorbance values higher than the cut-off were positive for anti-E2.

RESULTS

Physiological Changes

There were no abnormal physiological changes before or after infection in the animals. Macaques #14 and #11, who had mild hepatitis in their hepatocytes, were diagnosed by liver biopsy and also did not show any abnormal biochemical changes.

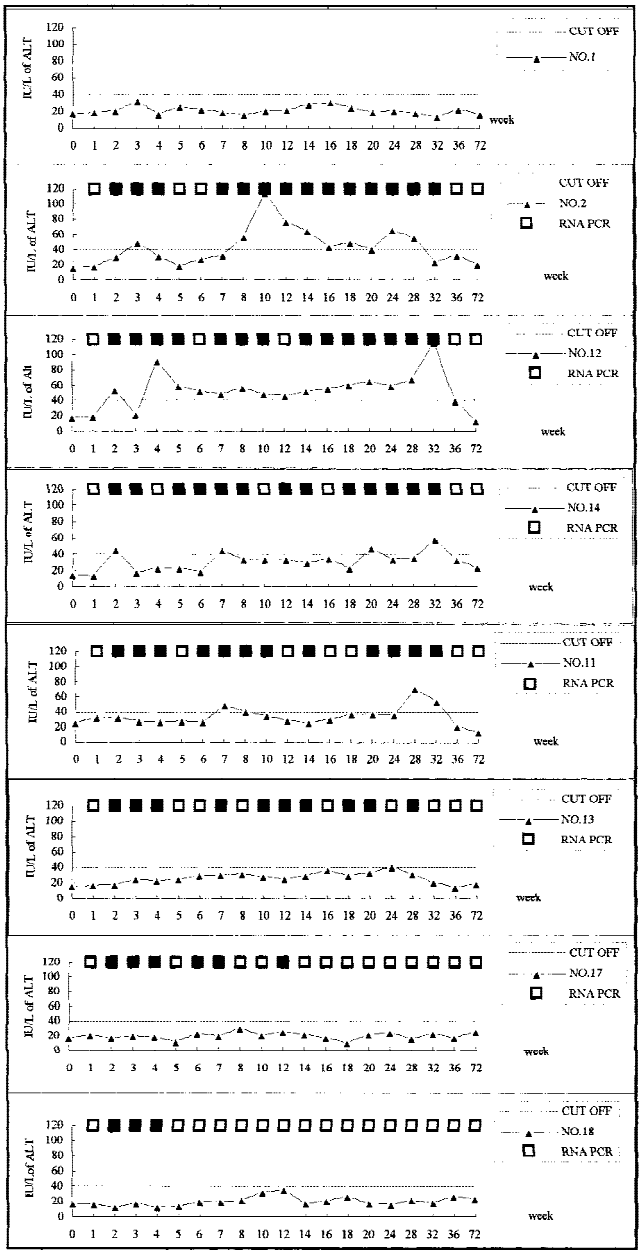

Fig. 2. (ALT) levels and (GBV-C/HGV) RNA in inoculated macaques: (■/□, positive/negative of GBV-C/HGV RNA; ▲ ALT levels). The cut-off level of ALT was 40 IU/L.

ALT Levels

All the inoculated macaques had a normal ALT level at 15–26 IU/L prior to inoculation. The dates were shown in Figure 2. There was a normal ALT level before or after inoculation in the control macaque #1. In macaque #2, the ALT level was elevated to 56 IU/L in the third week after inoculation and maintained more than 9 months at a level above 40 IU/L, the peak was about 116 IU/L at the 10th week. In macaques #12 and #14, the ALT levels were elevated above 40 IU/L at the second week and had a peak about 120 IU/L in ma-

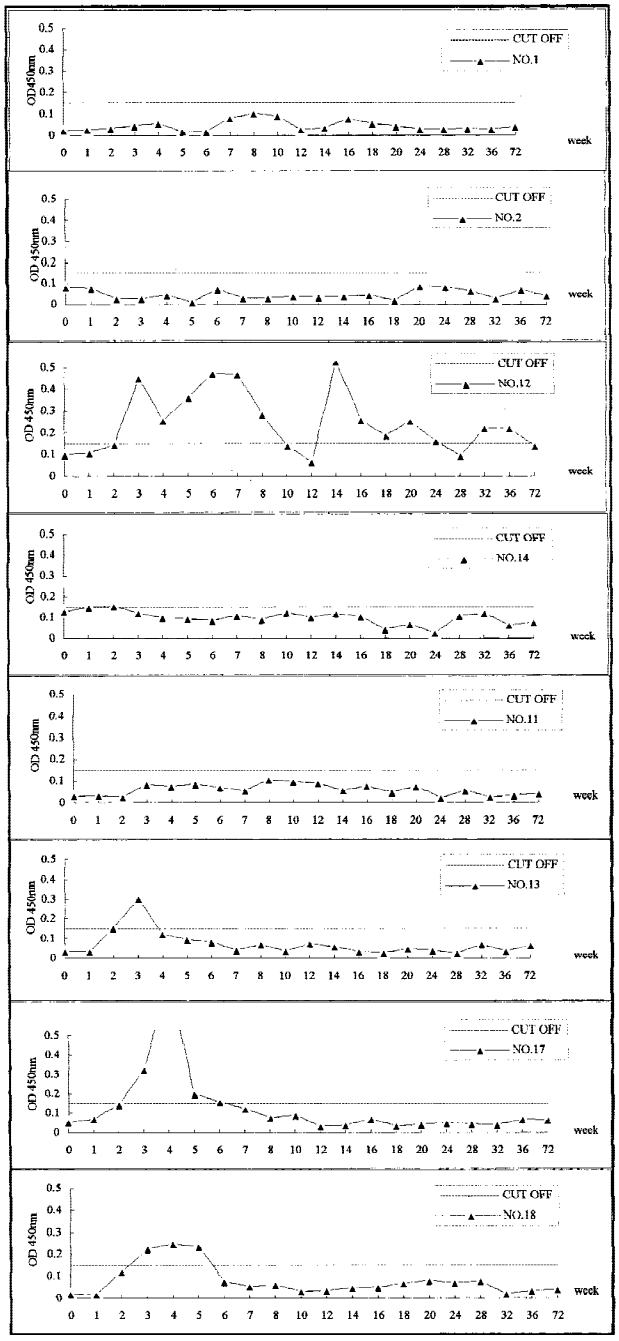

Fig. 3. Anti-E2 levels in the inocula. The cut-off value = Average OD_{450nm} of negative control macaque #1 plus 4SD; mean = 0.04505; SD = 0.02445. Cut-off = 0.14285.

caque #12 and 60 IU/L in macaque #14, respectively, at the 32nd week. The average ALT level in macaque #12 was higher than in macaque #14. For macaques #11 and #13, the ALT level in macaque #11 was elevated above 40 IU/L at the 7th week and had a peak about 76 IU/L at the 28th week; the ALT level was normal in macaque #13. The ALT levels in macaques #17 and #18 were normal. All the inoculated macaques had normal ALT levels after the 48th week.

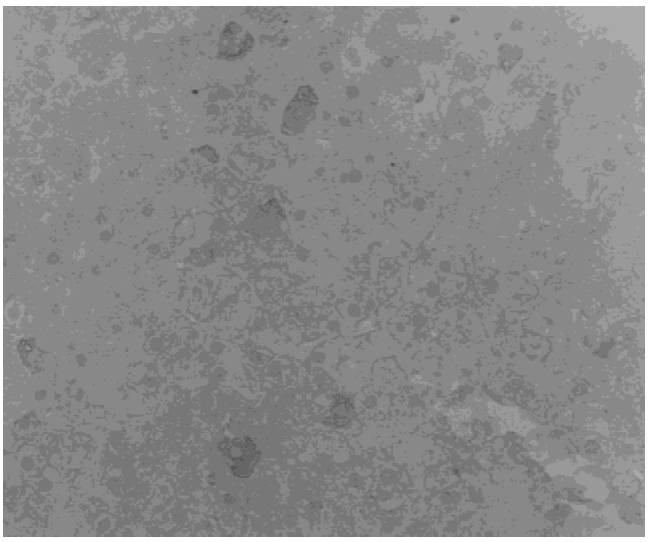

Fig. 4. The expression of NS5 antigen of GBV-C/HGV in hepatocytes of macaque #2 was positive by immuno-histochemistry (immuno-histochemistry stain 200×). The NS5 antigen-positive cell was sparsely distributed in liver tissue.

GBV-C/HGV-RNA

The RT-PCR was used for monitoring viremia associated with the GBV-C/HGV infection. All of the inoculated macaques had developed a viremia with GBV-C/HGV RNA in the second week postinoculation. The viremia persisted for 2–32 weeks in most macaques except macaque #18. The titers of the viremia were about 10^{3-4} diluted times before and after inoculation. Results are shown in Figure 2.

Anti-E2

The anti-E2 in the sera from the inocula was detected by using the synthesized-peptide ELISA. The positive anti-E2 were calculated based on the cut-off values plus 4 SD. The results are shown in Figure 3. Anti-E2 were not detected by ELISA in the control macaque (#1) and in the first inoculated macaque (#2). But there was a persisting positive anti-E2 detectable in the second passage of macaque #12. The anti-E2 in macaque #12 was positive from the third week and persisted for more than 72 weeks. The anti-E2 in second passage of macaque #14 and in the third passage of macaque #11 were negative. The anti-E2 became positive from the third week on in macaques #13, #17, and #18 and persisted for about 3 weeks.

Pathology

The histological appearances were normal (except for #14 and #11) in the inocula prior to challenge. The mild and acute hepatitis changes in five macaques appeared in the fifth week and became a mild chronic hepatitis during 36 weeks after inoculation except in macaques #11 and #14. Diffused swelling appeared in the mild inflammatory hepatocytes. The spotty necrosis and the infiltration of inflammatory cells were also found in the portal areas. The pathological changes in the hepato-

TABLE I. Pathological Changes in Hepatocytes of the Infected Macaques

Macaque (name)	Time postinfection (weeks)	Pathology[a]	Immunohistochemistry	In situ hybridization
2	0	Normal	–	–
	5	Mild AH	++	++
	36	Mild CH	+	+
	72	Very mild CH	–	–
12	0	Normal	–	–
	5	Mild AH	++	++
	36	Very mild CH	+	+
	72	Normal	–	–
14	0	Mild hepatitis	–	–
	5	Mild hepatitis	+	+
	36	Mild CH in porta area	–	–
	72	Mild CH in porta area	–	–
11	0	Mild hepatitis	–	–
	5	Mild hepatitis	+	+
	36	Very mild hepatitis	–	+
	72	Very mild hepatitis	–	+
13	0	Normal	–	–
	5	Mild AH	+	–
	36	Mild CH in porta area	–	+
	72	Very mild CH	–	–
17	0	Normal	–	–
	5	Mild AH	+	+
	36	Very mild CH	+	+
	72	Very mild CH	+	–
18	0	Normal	–	–
	5	Mild AH	+	+
	36	Mild CH	+	–
	72	Normal	–	–

AH, acute hepatitis; CH, chronic hepatitis.
There was very mild hepatitis in [14] and [11] macaques before inoculation and the pathogens were unknown.

cytes became milder (macaques #s 2, 14, 11, 13, and 17) or normal (#s 12 and 18) when the period of infection was prolonged. The results are shown in Figure 4 and Table I.

Immunohistochemistry and In Situ Hybridization in Hepatic Tissue

The immunohistochemistry and the in situ hybridization in hepatic tissues were negative before inoculation. The NS5 antigen and NS5 genome of the GBV-C/HGV in the hepatocytes became positive after inoculation. The positive results appeared in the 5th or 36th week and became negative 72 weeks after inoculation, except in macaques #11 and #17. NS5 antigen-positive hepatocytes were distributed sparsely in liver lobules. NS5 genome was detected only in cytoplasm of the antigen-positive or a few antigen-negative hepatocytes. There was no direct relationship between hepatitis and positive findings by immuno-histochemistry and in situ hybridization. The results are shown in Figures 4 and 5.

DISCUSSION

It is highly controversial whether or not the GBV-C/HGV contributed to the hepatitis agent. Evidence to date does not support the claim that GBV-C/HGV is responsible for non-A-E viral hepatitis. Accumulated reports appear to show that GBV-C/HGV may be an innocent bystander in a process caused by a non-A-E agent or some nonviral event [Alter, 1996; Loya, 1996]. Despite the lack of evidence implicating GBV-C/HGV in the pathogenesis of hepatitis, the presence of the virus in blood donations and in some hepatitis patients is clear. Little is known about the transmission and disease-inducing capacity of GBV-C/HGV. Many studies have also proved that GBV-C/HGV is a common, parenterally transmitted virus. More recently, tamarins and chimpanzees were used for the transmission of the GBV-C/HGV [Bukh et al., 1998] to determine their susceptibility to this virus. The conclusion was that "the chimpanzees, but not tamarins, were susceptible to GBV-C/HGV infection." In our report, the experimental macaques were susceptible to the infection with GBV-C/HGV.

The seven macaques were inoculated with blood from a non-A-E hepatitis patient as the infectious animal models. The serum levels of ALT, RT-PCR, anti-E2, pathology, immuno-histochemistry, and in situ hybridization were used to monitor the pathological changes of the hepatic tissues and the viremia associated with the GBV-C/HGV virus in the transmission study. The transmissibility of the GBV-C/HGV was demonstrated in these seven inoculated macaques. From this experiment, it appears that the GBV-C/HGV was possibly the cause of elevations in liver enzyme levels, as seen in macaques #s 2, 12, 14, and 11. No elevations of liver enzyme levels were observed in macaques #s 13, 17, 18, and control 1 after challenge.

GBV-C/HGV RNA became positive in the second or third week and lasted for 2–32 weeks after infection. There appears to be a relationship between the eleva-

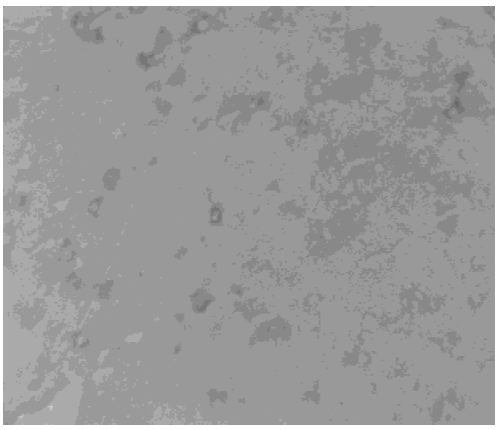

Fig. 5. The NS5 RNA was positive in the cytoplasm around the nuclei of hepatocytes of macaque #2 by in situ hybridization (DIG-labeled NS5 fragment as the probe, 200×). The NS5-RNA positive cell was sparsely distributed in liver tissue.

tion of liver enzyme levels and GBV-C/HGV RNA in sera during the first 2–3 weeks but it appeared unrelated longer after infection, especially for macaques #17 and #18. There appears to be relationships among the results of the immuno-histochemistry and in situ hybridizations in the hepatocytes and the positivities of the GBV-C/HGV RNA in the sera of these inoculated macaques. But these results appear to be unrelated to the damage of the hepatocytes. The immuno-histochemistry and the in situ hybridization results showed the possibility of NS5 protein expression and the viral genomic replication of GBV-C/HGV in the cytoplasm of the infected hepatocytes. Antibodies to E2 may have been responsible for resolution of the infection. Anti-E2 in inoculated macaques was detected by using synthesized-peptide EIA. The three inoculated macaques (#s 2, 14, and 11) that were infected persistently with viremia remained negative (in macaques #2 and #11) or at a low titer for antibodies to E2 (in macaque #14). But in macaques #s 12, 13, 17, and 18, anti-E2 appeared in the third week and persisted for about 3 weeks at a higher titer, except in macaque #12. The anti-E2 in macaque #12 was positive for nearly 72 weeks, although the anti-E2 was negative for a short time during the infectious period. There may be a relationship between the elevated ALT level and the appearance of anti-E2 in the infected macaques. It is possible that the antibodies to E2 may play an important role in the recovery of the infection, so that the clearance of the virus was accompanied with some injury of the liver cells. Macaques #s 2, 11, and 14 may have remained negative for anti-E2 because of some immunodeficiency in their immune system. Further research for the roles of the anti-E2 in the infected host is necessary.

Two macaques (#s 11 and 14) had a mild hepatitis before inoculation. The damage of the hepatocytes was not severe and the serum ALT levels were not elevated after inoculation, although the pathogenesis remains unknown. These results suggest strongly that GBV-C/HGV is the probable causative agent of hepatitis in the infected macaques and may become very mild or harmless to the hepatocytes of the host with time after the infection.

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